Published in final edited form as:
J Microsc. 2008 May ; 230(Pt 2): 263–267. doi:10.1111/j.1365-2818.2008.01983.x.

Silver enhancement of Nanogold particles during freeze substitution for electron microscopy

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Summary

Recent advances in rapid freezing and fixation by freeze-substitution have allowed structural cell biologists to apply these reliable modes of sample preparation to a wide range of specimens and scientific problems. Progress in electron tomography has produced cellular images with resolution approaching 4 nm in 3D, but our ability to localize macromolecules in these well-fixed, well-resolved samples has remained limited. When light fixation and low temperature embedding are employed with appropriate resins, immuno-localizations can recognize antigens at a section's surface, but labeling is therefore confined, not throughout the section's depth. Small, electron-dense markers, like Nanogold®, will often enter a living cell, serving as reliable tracers for endocytic activity, but these markers are usually too small to be visible in the context of a cell. We have developed a method for the silver enhancement of Nanogold particles that works during freeze substitution in organic solvents at low temperature. Here, we describe the development of this method, based on *in vitro* tests of reagents and conditions. We then show results from application of the method to an in vivo system, using Nanogold to track the internalization of immunoglobulin by neonatal murine intestinal epithelium, a specific example of receptor-mediated membrane traffic.

Keywords

endocytosis; immunogold; immunoEM; cytochemistry

Introduction

The localization of macromolecules by electron microscopy (EM) is of critical importance to understanding the relationships between cellular structure and function. To obtain reliable correlations between cytochemical labels with biological structure, however, the procedures for specimen preparation must produce an accurate representation of the specimen as it was in life. Rapid freezing, in conjunction with freeze substitution fixation and plastic embedding, can preserve cells and tissues in a manner that is superior to the more traditional methods of chemical fixation (Bridgman and Reese, 1984; Heath and Rethoret, 1982; Howard and O'Donnell, 1987). High pressure freezing allows one to cryo-immobilize cellular samples up to 300 μm thick without the formation of damaging ice crystals,

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providing a versatile and reliable method for cryo-immobilization of diverse cellular specimens (Moor, 1987; McDonald and Morphew, 1993; Sartori et al., 1993; Studer et al., 2001). Such samples can be dehydrated at low temperature, using a solvent like acetone that displaces water efficiently at −90°C, greatly improving the preservation of cellular detail relative to that which is seen after dehydration in a solvent concentration series at 0°C (Kellenberger, 1991). Chemical fixatives or stains can be dissolved in the solvent used for freeze-substitution, providing a measure of cross-linking and contrast enhancement to improve sample preservation for EM imaging (Hess, 2007; McDonald, 2007). Samples can then be embedded in plastic, sectioned and imaged, with either conventional 2D methods or in 3D through tomographic reconstruction from a series of tilted views (McIntosh et al., 2005). The structural detail that is visible in these tomograms is impressive and can help characterize functionally important facets of cellular organization.

Such structural maps are, however, not sufficient to localize specific macromolecules or to track specific pathways of membrane traffic, e.g., during receptor-mediated endocytosis. Nanogold is an appropriate marker for studies of fluid phase or receptor-mediated transport across the cell membrane because it is relatively small and can be linked covalently to specific ligands (Hainfeld and Powell, 2000). However, these small gold particles are not easily seen within cellular samples of a thickness appropriate for electron tomography; they must be enlarged by methods that grow grains of silver or gold around the small gold probe (Lah et al., 1990; Shah et al., 1995; Weipoltshammer et al., 2000). Currently, reliable reagents and methods for silver “enhancement” (i.e., enlargement) are available as kits from commercial vendors <http://www.nanoprobes.com/> or can be made on the lab bench (Danscher et al., 1995). These methods, however, rely on aqueous environments that are not compatible with the organic solvents and the low temperatures used during freeze substitution. We have developed a stepwise, *in vitro* protocol for visualizing the effectiveness of various protocols for the silver enlargement of Nanogold and have used it to identify enhancement conditions that are compatible with the solvents used in freeze-substitution. Here we describe the method and its application to an in vivo system of cellular membrane trafficking.

Materials and Methods

In vitro development of silver enhancement

Preparation of enhancement reagents—Reagents for silver development were prepared in separate tubes as saturated solutions of silver nitrate (0.04%), hydroquinone (0.3%), citric acid (0.4%) and sucrose (0.1%) in pure acetone. Solutions were protected from light and mixed on a rocking platform for 4 hours, then cooled to −20°C overnight. Cooled solutions were centrifuged at 3000RPM for 10 min to remove insoluble material, then cooled to −50°C, and appropriate volumes of the four components were mixed in pre-cooled containers for conducting tests.

Assays on Blotter paper—10 μl of undiluted Nanogold® solution (Nanoprobes, Inc., Yaphank, NY) was applied to small strips (1cm × 3cm) of nylon blotting paper (Boehringer Mannheim) and allowed to air dry. Paper strips were soaked in various ratios of the four enhancement components overnight at temperatures ranging from 4°C to −50°C. Strips were rinsed in acetone at the respective temperature and evaluated based on color.

Assays on EM Grids—5 μl of undiluted Nanogold solution was applied to Formvar-coated, carbon-stabilized EM grids and allowed to air dry. Grids were soaked overnight, as described above for blotter paper, rinsed in cold acetone and air dried. Grids were examined on a Tecnai F20 electron microscope (FEI Co., Hillsboro, OR) operating at 200 kV.

Assays on Lysed cells—Ptk_1 cells were grown by standard methods (Bridgman and Reese, 1984) on Formvar-coated, carbon stabilized EM grids. After they had reached ~70% confluence, the cells were detergent-extracted in 0.1% Triton-X100 in Pipes buffer, then fixed with paraformaldehyde and glutaraldehyde as previously described (Schliwa, 1987). Fixed cells were incubated in antibodies against tubulin followed by goat anti-mouse IgG conjugated to Nanogold particles (Nanoprobes, Inc.). Grids with labeled cells were dehydrated into acetone by progressively lowering the temperature while raising the concentration of the solvent to 100% acetone at −50°C (Carlemalm et al., 1985). Grids were soaked overnight at −50°C in the enhancement solution. Some were then rinsed with acetone at −50°C while others were allowed to warm to −30°C before rinsing. All grids were dried by the critical point method (Ris, 1985) and examined in an FEI Tecnai F20 electron microscope operating at 200kV.

In vivo enhancement of Nanogold

Specimens—Nanogold particles conjugated to the Fc moiety of IgG were fed to newborn rats (He et al., 2007). Briefly, 200–300μl of rat Fc Nanogold conjugates at ~2μM in 20 mM $NaPO_4$, 1.2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.25 mM $MgSO_4$, pH 6.0 at 37°C were fed to 12-day-old Sprague Dawley rats that had fasted 3 hours before feeding. After 120 minutes, the rats were anesthetized with CO_2, sacrificed, and a small segment of the duodenum was excised, cut into pieces (2 mm), transferred to freezing planchettes (Engineering Office M. Wohlwend), and frozen in a Balzers HPM010 high pressure freezer (Bal-tec AG, Liechtenstein). Hexadecene was used as a filler to occupy empty space within the planchette-specimen sandwich.

Freeze substitution—Frozen planchettes were separated using the blade of a cooled #11 scalpel blade and transferred under liquid nitrogen to vials containing a frozen solution of 2% glutaraldehyde and 0.01% tannic acid dissolved in acetone. Vials with sample were transferred to a pre-cooled Leica freeze substitution machine (Leica EM AFS, Vienna) at −90°C for 2 days. Samples were warmed to −50°C over 6 hours and were rinsed three times with cold acetone.

Silver enhancement—The four components necessary for silver enhancement of small gold were prepared as above. Cooled components were mixed by adding 2 parts silver nitrate, 2 parts hydroquinone, 1 part citric acid and 1 part sucrose. The mixture was then cooled to −50°C in the dark and added to each 1.5 ml sample vial. After 12 hours at −50°C, samples in enhancement solution were warmed to −30°C over 6 hours, rinsed 3× in cold acetone, post-fixed with 0.5% glutaraldehyde containing 0.1% uranyl acetate, warmed to room temperature over 6–12 hours and rinsed in acetone.

Sectioning and microscopy—Samples were infiltrated with epoxy resin, and tissue pieces were flat-embedded between two treated microscope slides by a method previously described (Reymond and Pickett-Heaps, 1983). After polymerization of the resin, small pieces of embedded tissue were excised and remounted onto blank epoxy stubs. Ribbons of 300 nm sections were collected on Formvar-coated copper slot grids and post-stained with uranyl acetate and Reynold's lead citrate. Sections were imaged in an FEI Tecnai F30 microscope operating at 300 kV. Areas containing the intestinal lumen were imaged through serial tilts ±60° and tomographic reconstructions were computed as previously described (Mastronarde, 1997).

Results

In vitro enhancement

Initial enhancement experiments on blotting paper showed that it was possible to precipitate and develop silver grains in acetone, despite the relatively low solubility of the necessary reagents in anhydrous solutions. Our first tests were at 4°C and demonstrated rapid development of silver (1 hour), showing dark spots where Nanogold had been applied. Different ratios of enhancement components produced spots of different shades (Figure 1a) with the biggest effect seen with greater amounts of the hydroquinone component. Similar testing at lower temperatures (−20°C and −50°C) also produced development on the blots, although the times required to see a result were longer (8–12 hours).

Results from these experiments were used to set up productive tests of Nanogold enhancement on EM grids. Our goal was to produce particles of fairly uniform size not larger than 8nm. We tested several ratios of silver nitrate: hydroquinone: citric acid but obtained the best results with 2:2:1 and temperatures from −20°C to −50°C. Particle growth and size was best observed when grids were developed at −50°C overnight, but warmed to −30°C for 6–8 hr before rinsing. The addition of one part saturated sucrose in acetone helped prevent the growth of extremely large particles, but they still ranged in size from 4–13 nm in these *in vitro* experiments (Figure 1b).

Lysed tissue culture cells provided a system with which to test the reliability of our development, and they provided a substrate to evaluate background nucleation of silver. By applying the same conditions that worked well for the grid method, as above, we were able to enhance Nanogold particles directed against tubulin without appreciable background nucleation (Figure 1c). Longer incubation times in the enhancing solutions, along with warmer temperatures, produced a much more dense decoration of microtubules, but the particles began to grow to 20 nm or more, a size unsuitable for our applications. Further, longer times begin to produce autonucleation background noise.

Enhancement in cellular samples

Nanogold uptake into intestinal epithelial cells was chosen as a useful system in which to test our method for the enhancement of gold in a cellular context. Intestinal tissue was processed as described in Methods. We avoided the use of osmium tetroxide or other heavy metals prior to the addition of enhancement components, because these acted as nucleation sites for silver growth (Dancher, 1981). Uranyl acetate was used post-enhancement to add contrast to the tissue. Since our application was for tomographic reconstruction, the tissue sections used were 300 nm thick. At this thickness, we were not able to visualize enhanced gold particles in single EM projections, but they were evident in slices of the computed tomogram (Figure 2a–d). In these cellular samples, particle size of enhanced Nanogold ranged from 3–8 nm even though we imposed the same conditions of solution composition, time and temperature as for the *in vitro* experiments, which produced 4–13 nm particles. A subset of the tomographic volume that contained enhanced gold was selected, then membrane contours (purple) and enhanced gold particles (yellow) were modeled, using the IMOD software package (Kremer et al., 1996) (Figure 3a,b). The model view through its 300 nm thickness showed the ability of the enhancement reagents to penetrate the tissues, even at low temperature.

Discussion

Silver enhancement of Nanogold particles in an organic solvents during freeze substitution at low temperature represents a useful advance for the localization of some taggable proteins in well-fixed samples by tomographic reconstruction. The use of blotting paper (a useful

recommendation by Dr. Richard Burry) allowed us to test a number of temperatures and combinations of reagents quickly and easily. This simple evaluative method could also be applied to test a number of novel labeling techniques.

Although our procedure does precipitate silver onto Nanogold within a volume of tissue, there are issues that suggest routes for further improvement. Our aim was to produce particles no larger than 8 nm, so we could retain structural information relative to the label. When this size limit was enforced, not all of the Nanogold particles within the tissue were visibly enhanced, particle size was not uniform and we were not able to see the particles in 2-D projections of 300 nm sections. The enhancement solution was able to penetrate deep into the sample to nucleate silver onto Nanogold particles which were taken up during endocytosis but the enhanced particles resulting after tissue penetration were smaller, on average, than those observed during *in vitro* tests. Further, the fact that we did not add osmium during the primary fixation limited the preservation of membranes in the sample. Nonetheless, our tissue samples appear nicely preserved, even though the freeze-substitution solution did not contain osmium. We therefore think that others may find this combination of silver enhancement with small gold labeling and rapid freezing, freeze substitution fixation a useful method for high quality labeling work in the electron microscope.

Acknowledgments

We gratefully acknowledge Eileen O'Toole for assistance with modeling. This work was supported in part by RR00592 from the NIH to JRM and 2R37 AI041239-06A1 to P.J.B.

References

Bridgman PC, Reese TS. The structure of cytoplasm in directly frozen cultured cells. I. Filamentous networks and cytoplasmic ground substance. J.Cell Biol. 1984; 99:1655–1668. [PubMed: 6436253]

Carlemalm E, Villiger W, Hobot JA, Acetarin JD, Kellenberger E. Low temperature embedding with Lowicryl resins: two new formulations and some applications. J Microsc. 1985; 140:55–63. [PubMed: 3912508]

Danscher G. Histochemical demonstration of heavy metals. A revised version of the sulphide silver method suitable for both light and electronmicroscopy. Histochemistry. 1981; 71:1–16. [PubMed: 6785259]

Danscher, G.; Hacker, GW.; Hauser-kronberger, C.; Grimelius, L. Trends in autometallographic silver amplification of colloidal gold particles. In: Hyatt, MA., editor. Immuno-gold staining: Principles, methods, and applications. Boca Ratom, FL: CCR Press; 1995. p. 11-18.

Hainfeld JF, Powell RD. New frontiers in gold labeling. J Histochem Cytochem. 2000; 48:471–480. [PubMed: 10727288]

He W, Kivork C, Machinani S, Morphew MKAM, Gail AM, Tesar DB, Tiangco NE, McIntosh JR, Bjorkman PJ. A Freeze Substitution Fixation-Based Gold Enlarging Technique for EM Studies of Endocytosed Nanogold-Labeled Molecules. J. Struct. Biol. 2007 submitted.

Heath IB, Rethoret K. Mitosis in the fungus Zygorhynchus moelleri: evidence for stage specific enhancement of microtubule preservation by freeze substitution. Eur.J.Cell Biol. 1982; 28:180–189. [PubMed: 7173217]

Hess, MW. Cryopreparation Methods for Electron Microscopy of Selected Model Systems. In: McIntosh, JR., editor. Cellular Electron Microscopy. Vol. vol. 79. San Diego: Academic Press; 2007. p. 57-97.

Howard RJ, O'Donnell KL. Freeze substitution of fungi for cytological analysis. Exptl.Mycology. 1987; 11:250–269.

Kellenberger E. The potential of cryofixation and freeze substitution: observations and theoretical considerations. J.Microscopy. 1991; 161(Pt 2):183–203.

Kremer JR, Mastronarde DN, McIntosh JR. Computer visualization of three-dimensional image data using IMOD. J Struct Biol. 1996; 116:71–76. [PubMed: 8742726]

Lah JJ, Hayes DM, Burry RW. A neutral pH silver development method for the visualization of 1-nanometer gold particles in pre-embedding electron microscopic immunocytochemistry. J Histochem Cytochem. 1990; 38:503–508. [PubMed: 2319121]

Mastronarde DN. Dual-axis tomography: an approach with alignment methods that preserve resolution. J.Struct.Biol. 1997; 120:343–352. [PubMed: 9441937]

McDonald, K. Cryopreparation Methods for Electron Microscopy of Selected Model Systems. In: McIntosh, JR., editor. Cellular Electron Microscopy. Vol. vol. 79. San Diego: Academic Press; 2007. p. 23-55.

McDonald K, Morphew MK. Improved preservation of ultrastructure in difficult-to-fix organisms by high pressure freezing and freeze substitution: I. Drosophila melanogaster and Strongylocentrotus purpuratus embryos. Microsc.Res.Tech. 1993; 24:465–473. [PubMed: 8490232]

McIntosh R, Nicastro D, Mastronarde D. New views of cells in 3D: an introduction to electron tomography. Trends Cell Biol. 2005; 15:43–51. [PubMed: 15653077]

Moor, H. Theory and practice of high pressure freezing. In: Steinbrecht, RA.; Zierold, K., editors. Cryotechniques in Biological Electron Microscopy. Springer Verlag; 1987. p. 175-191.

Reymond OL, Pickett-Heaps JD. A routine flat embedding method for electron microscopy of microorganisms allowing selection and precisely orientated sectioning of single cells by light microscopy. J Microsc. 1983; 130:79–84. [PubMed: 6343615]

Ris H. The cytoplasmic filament system in critical point-dried whole mounts and plastic-embedded sections. J Cell Biol. 1985; 100:1474–1487. [PubMed: 4039327]

Sartori N, Richeter K, Dubochet J. Vitrification depth can be increased more than 10-fold by high-pressure freezing. J.Microscopy. 1993; 172:55–61.

Shah N, Zhang S, Harada S, Smith RM, Jarett L. Electron microscopic visualization of insulin translocation into the cytoplasm and nuclei of intact H35 hepatoma cells using covalently linked Nanogold-insulin. Endocrinology. 1995; 136:2825–2835. [PubMed: 7789307]

Studer D, Graber W, Al-Amoudi A, Eggli P. A new approach for cryofixation by high-pressure freezing. J Microsc. 2001; 203:285–294. [PubMed: 11555146]

Weipoltshammer K, Schofer C, Almeder M, Wachtler F. Signal enhancement at the electron microscopic level using Nanogold and gold-based autometallography. Histochem Cell Biol. 2000; 114:489–495. [PubMed: 11201611]

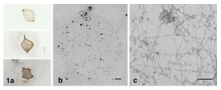

Figure 1.

Precipitation and development of silver grains in acetone. (a) Nanogold applied to blotter paper and developed in different ratios of enhancement components at various times and temperatures may be evaluated based on color change. Darker spots indicate most reactive conditions. (b) Nanogold particles applied to formvar-coated EM grids then silver enhanced using saturated solutions of silver nitrate, hydroquinone, citric acid and sucrose in acetone (2:2:1:1). Enhanced particles range in size from 4–13 nm. Bar=40 nm (c) Ptk cells labeled with antibodies to tubulin and secondary antibodies conjugated to nanogold then silver enhanced. Enhanced Nanogold particles are clearly visible along microtubules with little background staining. Bar=0.4 um

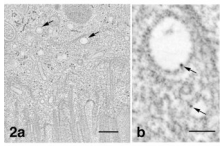

Figure 2.

Nanogold uptake into intestinal epithelial cells. (a) A 6 nm tomographic slice through a portion of intestinal tissue. Conjugated Nanogold particles were fed to rats and intestinal tissue was high pressure frozen, freeze substituted into acetone containing glutaraldehyde and tannic acid at −90°C, warmed to −50°C and silver enhanced as described. 300 nm thick sections were imaged through serial tilts on a Tecnai F30 and a tomographic reconstruction was computed. Under these conditions, enhanced particle (arrows) size ranged from 3–8 nm. Bar=0.25 um (b) Higher magnification of the 5 nm tomographic slice show enhanced particles (arrows) inside vesicular components of the tissue. Bar=0.1 um.

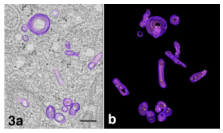

Figure 3.
(a) Selected membrane contours (purple) and enhanced gold particles (yellow) were modeled through a subset of the tomographic volume using the IMOD software package. Bar=0.2 um (b) A 3D model view of selected membrane components show the distribution of particles within membranes through the 3D volume.